Detection of Added Whey Protein Concentrate in Nonfat Dry Milk by Amino Acid Analysis

Existing procedures for the examination of nonfat dry milk (NDM) to detect added whey protein concentrate (WPC) are time consuming or require a great deal of sample handling. Amino acid analysis of NDM acid hydrolyzates with computer data handling is a method amenable to automation that will detect levels of >10% added WPC. Values in microgram percent for the amino acids aspartic acid, alanine, and proline are used as markers both in the screening procedure for simple acceptance or rejection of NDM samples and if desired the quantitative estimation of the extent of adulteration. The amino acid analysis results are not affected by heat treatment used in the NDM drying procedure, and the method is valid whether the WPC source is acid or sweet whey.

INTRODUCTION

Nonfat dry milk (also called skim milk powder) is widely used in the food industry and is a component of a wide spectrum of manufactured foods including baked goods, dairy products, and many other processed foodstuffs. Whey protein concentrates, produced in larger quantities in recent years as a result of changes in whey disposal practices, have a more limited commercial application and are available at a lower price. This makes it financially attractive to contravene federal regulations (Code of Federal Regulations, 1983) and adulterate nonfat dry milk (NDM) with whey protein concentrate (WPC). Since the nutritional parameters and functional properties of WPC differ from those of NDM (80% casein, 20% whey), it is important for regulatory agencies and food manufacturers to be able to detect such additions. Monitoring this adulteration is not a simple problem, since some WPC is formulated to be isoprotein and isolactose with NDM.

Neither the traditional Harland–Ashworth (1947) turbidimetric method for determination of undenatured whey proteins in heat-treated milk or the modification by Leighton (1962) can be successfully applied to quantitate WPC blended with NDM (Basch et al., 1985). Olieman and van den Bedem (1983) and van Hooydonk and Olieman (1982) developed a method for determining the amount of rennet (sweet) whey total solids in NDM by HPLC measurement of the glycomacropeptide (GMP) present. This procedure requires considerable "wet chemistry" (i.e., precipitation and filtration for each sample) and will not detect added acid whey powder produced by direct acidification. The gel electrophoretic procedure for whey quantitation reported by Basch et al. (1985) involves many manipulations, and there is a 3-day time factor for obtaining the results. Since the caseins that comprise the major protein fraction of NDM are quite low in cystine content, the finding of significant quantities of this amino acid is indicative of the presence of whey protein. Polarographic measurement of cystine (Mrowetz and Klostermayer, 1976) has therefore also been applied as a threshold index of added whey protein.

It is clear that there is a need for a routine screening procedure for detection and quantitation of added WPC in NDM, a procedure requiring a minimum of sample handling and amenable to automation. During extensive storage studies of NDM (Greenberg et al., 1977), we have observed that the amino acid profile with the exception of several sensitive amino acids is remarkably constant and is independent of the heat treatment used in the drying procedure. This report presents a method satisfying the aforementioned criteria and based on automatic amino acid analysis, which will permit the detection and/or quantitation of added WPC in NDM in a timely manner.

MATERIALS AND METHODS

Dry Milk and Whey. Skim milk powders (NDM) were sampled from lots stored by ASCS, USDA, in various locations throughout the U.S. The low- and high-heat NDM

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standards were supplied by the American Dry Milk Institute, Chicago, IL. Whey protein concentrate powders (WPC) were commercial samples obtained from the AMS Testing Laboratory, Chicago, IL.

Sample Preparation. Duplicate or triplicate 1–3-mg samples of NDM or WPC were hydrolyzed at 110 °C with 1.0 mL of 5.7 N HCl containing phenol (0.05%) for 24 h in tubes evacuated to 20 μm and sealed. After hydrolysis, the HCl was removed by rotary evaporation or by use of a heated Savant Speed Vac concentrator. Samples were syringe filtered before analysis.

Performic Acid Oxidation. The method of Hirs (1956) as modified by Moore (1963) was employed for conversion of cystine to cysteic acid.

Amino Acid Analysis. Hydrolyzate aliquots containg ca. 50 μg of protein were analyzed on a Beckman 119CL amino acid analyzer interfaced with a Hewlett-Packard 3390A integrator. Up to 30 samples can be loaded for unattended operation. The standard protein hydrolyzate procedure with a 0.6 × 22 cm column of W3H ion-exchange resin and a 96-min total analysis time was employed. Details: citrate buffers, pH 3.25, 0.20 N Na^+, changed to pH 3.95, 0.40 N Na^+, at 26 min and to pH 6.4, 1.00 N Na^+, at 44 min. Regeneration at 79 min for 2 min with 0.2 N NaOH is then followed by reequilibration with starting buffer for 15 min. The program includes a column temperature change from 50 to 65 °C at 20 min. A standard mixture containing 10 nmol of each amino acid was included with each batch of samples analyzed.

Data Handling. The integrator output (nanomoles of each amino acid) served as the input for a computer program that can calculate for each amino acid micrograms/sample, microgram percent, micromoles/100 micromoles, and weight percent. Amino acid composition expressed as microgram percent, proved the most useful for this study.

RESULTS AND DISCUSSION

Cystine Determination. Differences in the cystine content of WPC (2.95 ± 0.7 μg %) and NDM (0.98 ± 0.04 μg %), measured as cysteic acid by amino acid analysis after performic acid oxidation and hydrolysis, are certainly substantive and could serve as an index of adulteration. Cysteic acid elutes as the first peak (ca. 6 min) during ion-exchange amino acid analysis and in this system is not subject to variability caused by ion front effects. This allows a significant reduction of analysis time if determination of the neutral and basic amino acids is sacrificed and early regeneration instituted. Under these conditions, the microgram percent calculation reported above would be replaced by the weight percent value, which tends to be less accurate due to weighing and diluting small samples. However, despite the reproducibility of the microgram percent results and possible reduction of analysis time if the shortened method is chosen, the performic acid oxidation procedure is much too cumbersome for multisample use and does not satisfy the criterion of limited sample handling.

Total Amino Acid Composition. To test our observation that the amino acid composition of NDM is remarkably constant and independent of the source, heat treatment, and conditions or length of storage, samples varying in these parameters were collected from warehouses. These materials along with standard low- and high-heat powders were hydrolyzed with HCl and analyzed by the procedure described in Materials and Methods. As can be observed in the first three columns of Table I, there is good agreement among all the NDM samples; the average error of 3–5% is the error of the method. Comparable data for the WPC samples, the last column in Table I, are also precise with the same 3–5% standard error.

Table I. Amino Acid Composition of Nonfat Dry Milk and Whey Protein Concentrate (μg %)

amino acid	NDM[a]	NDM std[b] low heat	NDM std[b] high heat	WPC[c]
Asp	7.36 ± 0.37	6.81	6.90	10.63 ± 0.11
Thr	4.38 ± 0.22	4.45	4.32	6.94 ± 0.16
Ser	5.35 ± 0.41	5.14	4.81	4.73 ± 0.18
Glu	18.25 ± 0.63	18.68	18.71	16.35 ± 0.68
Pro	9.68 ± 0.41	10.20	9.89	6.34 ± 0.46
Gly	2.03 ± 0.07	2.03	1.92	1.99 ± 0.03
Ala	3.29 ± 0.13	3.34	3.23	4.81 ± 0.16
Cys	0.39 ± 0.19	0.63	0.75	1.62 ± 0.37
Val	5.95 ± 0.20	5.95	6.07	6.08 ± 0.17
Met	3.51 ± 0.45	2.77	3.18	3.06 ± 0.24
Ile	5.62 ± 0.35	5.43	5.78	7.16 ± 0.63
Leu	9.52 ± 0.27	9.13	9.27	10.63 ± 0.26
Tyr	5.39 ± 0.39	5.52	5.98	3.62 ± 0.53
Phe	4.90 ± 0.17	5.14	5.11	3.56 ± 0.17
His	3.22 ± 0.29	3.35	3.31	1.60 ± 0.28
Lys	7.64 ± 0.33	7.65	7.40	8.87 ± 0.45
Arg	3.53 ± 0.18	3.63	3.61	2.90 ± 0.20

[a] Mean ± standard error for 10 samples analyzed in duplicate. [b] Duplicate analyses for each standard sample. [c] Mean ± standard error for five samples analyzed in dupblicate.

Table II. Joint Confidence Intervals for the Detection of Adulteration

conf level, %	reject if Asp	reject if Ala	reject if Pro
95	>8.92	>3.60	<8.69
99.5	>9.25	>3.70	<8.39
99.8	>9.46	>3.80	<8.10

By inspection, aspartic acid (Asp), proline (Pro), and alanine (Ala) were chosen as key compounds to test as mixture "markers". These amino acids are not sensitive to the time of hydrolysis and differ in quantity by more than 5 standard deviations in the NDM and WPC comparison (Table I). A statistical model was then used based on the assumption that any mixture of NDM and WPC would yield values for these "markers" that would be a corresponding linear combination of the values from Table I. This is represented by

$$\tilde{x}_s = \alpha\tilde{x}_n + (1 - \alpha)\tilde{x}_w \quad (1)$$

where $\tilde{x}_s$ (Asp_s, Ala_s, Pro_s) is the amino acid content in microgram percent of a sample s consisting of 100α% NDM and $100(1 - \alpha)$% WPC, $\tilde{x}_n$ = 7.36, 3.29, and 9.68, and $\tilde{x}_w$ = 10.63, 4.81, and 6.34. In addition, a joint confidence interval (Table II) was constructed from the Table I aspartic acid, proline, and alanine values for various confidence levels. Any sample falling outside of an interval can be judged to be a mixture (adulterated) at the given confidence level, and the equations based on (1) can be used to obtain an estimate of the degree of adulteration.

Known mixtures containing 30, 50, 65, 80, and 90% NDM with the remainder WPC were prepared, analyzed, and classified as adulterated (accepted or rejected) according to Table II. At the 99.5% confidence level, all samples below 90% NDM (>10% WPC) were rejected. Thus, WPC added to NDM at a level above 10% would be detected. This range is equivalent to that reported for the GMP–HPLC method (Olieman and van den Bedem, 1983), and economics dictate that the practice of addition of lower levels of WPC to NDM is not likely to occur.

Three blends of NDM and WPC were prepared, hydrolyzed, and analyzed in duplicate, and the percent of each estimated by the alanine microgram percent data

Table III. Percent NDM in Blends with WPC

sample	known NDM	μg % Ala[a]	calcd NDM
I	88	3.54	84
II	43	4.19	41
III	77	3.75	70

[a] Average of duplicate runs.

Table IV. Percent WPC as Determined by the Gel Electrophoretic (PAGE) and Amino Acid Analysis Methods

sample	known % WPC	PAGE	amino acid
A	0	4	0
B	5	5	6
C	10	0	11
D	15	7	13
E	45	44	49
F	50	47	53
G	55	34	56
H	75	71	68

according to eq 1. These results as shown in Table III confirm that the linear model does produce acceptable quantitation. A sample calculation for the first entry would be as follows:

$$\alpha = (4.81 - 3.54)/1.52 = 0.84$$

Although alanine is used for this example, similar results are obtained with aspartic acid and/or proline.

Another set of NDM and WPC mixtures, prepared elsewhere, was examined by both the amino acid procedure and the gel electrophoretic–WPI method (Basch et al., 1985). The estimated degree of adulteration expressed as percent WPC for these samples is presented in Table IV. Below 15% WPC, both methods lose sensitivity, with the amino acid analysis procedure perhaps a better approximation. At higher levels, the data are comparable.

Quantitation of the amino acid "markers" to the necessary level of accuracy and calculation of microgram percent require the analysis system to be reproducible and capable of separating all the components of the hydrolyzate. A dedicated amino acid analyzer with an ion-exchange resin column, postcolumn ninhydrin detection system, and dual-wavelength (570, 440 nm) monitoring is optimal for use in a long-term screening program. New techniques involving precolumn derivatization, fluorescence detection, and reversed-phase HPLC (RP-HPLC) amino acid separation have several drawbacks. According to Elkin (1984) in his comparison of the two procedures as applied to feedstuff hydrolyzates, the ion-exchange procedure was less variable than the HPLC method. In addition, glycine and threonine were not resolved by RP-HPLC and therefore not calculated, and proline and cystine were not detected at all. The advantages of ion-exchange and postcolumn detection are well stated by Dong et al. (1985) who discuss a system coupling these features with the speed and sensitivity of HPLC. Reduction of the chromatographic analysis time from 96 to 30 min is certainly possible, but the total procedure must still include an overnight hydrolysis step.

Detection of WPC in NDM as described herein was carried out on a standard amino acid analyzer. It is a fact that acquisition of such dedicated instrumentation is costly, but in our experience the expenditure per analysis for buffers, ninhydrin, standards, etc., is less than $2.00, a figure that compares favorably with alternate procedures. Sample throughput with this system is 75–80/week; results for any one sample are available in a total time of 30 h including weighing, hydrolysis, evaporation, and analysis. The same ion-exchange resin has been in use in our laboratory for over 4500 analyses; it can be removed from the column, cleaned, and repacked without problem and with reasonable care should last many years. To maintain accurate quantitation, it is wise to prepare fresh dilutions of the amino acid standard mixtures every 2 weeks and include them at the beginning and end of every batch of samples. The computer output should monitor microgram percent values for the three "marker" amino acids (aspartic acid, alanine, proline) and flag any sample where one or more of these values lies outside the limits of Table II. Such suspect samples can be reanalyzed to confirm the addition of WPC or examined by an alternate procedure such as gel electrophoresis (Basch et al., 1985) or HPLC analysis of the GMP (Olieman and van den Bedem, 1983).

This screening procedure satisfies the criteria for limited sample handling and automation and will detect the addition of WPC to NDM whether the whey source is sweet and/or acid whey.

ACKNOWLEDGMENT

We thank Virginia H. Holsinger and James Dizikes for assistance in obtaining samples, John G. Phillips for the statistical analyses, and J. J. Basch for the polyacrylamide gel electrophoresis results.

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Received for review June 24, 1985. Accepted October 7, 1985. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.